Journal club presentation

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A New Role for ERα: Silencing via DNA Methylation of Basal, Stem Cell, and EMT Genes

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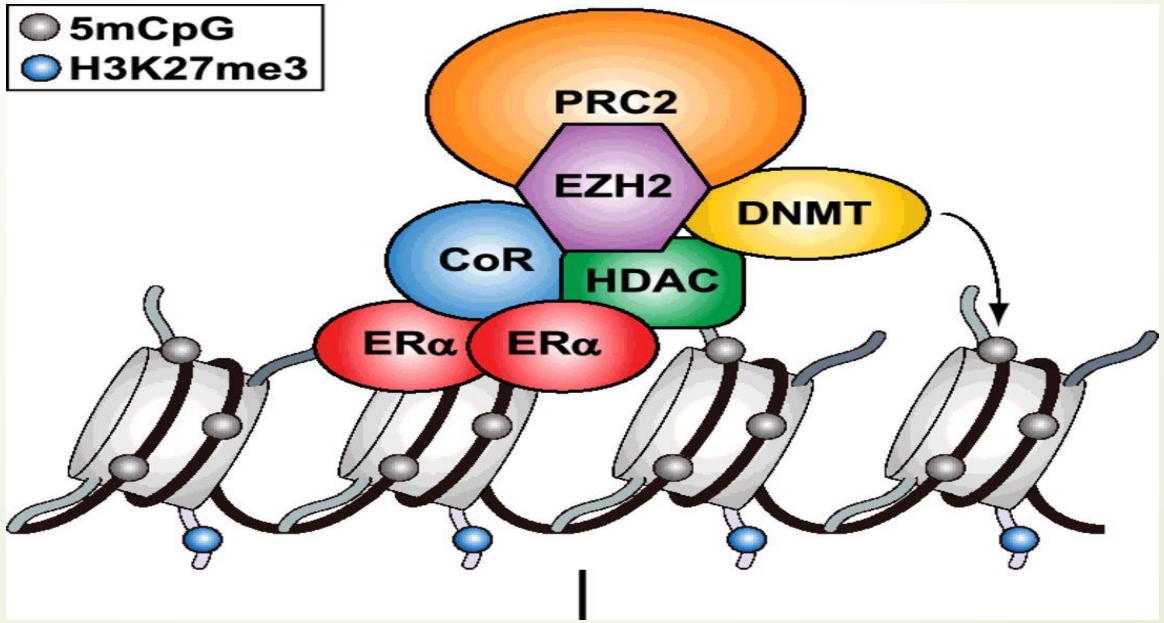


Abstract

- Resistance to hormonal therapies is a major clinical problem in the treatment of estrogen receptor α–positive (ERα+) breast cancers
- DNA methylation of cytosine at specific CpG sites are frequently associated with ERα+ status in human breast cancers
- Hypothesis : ERα may regulate gene expression via DNA methylation

Abstract

Evaluations :

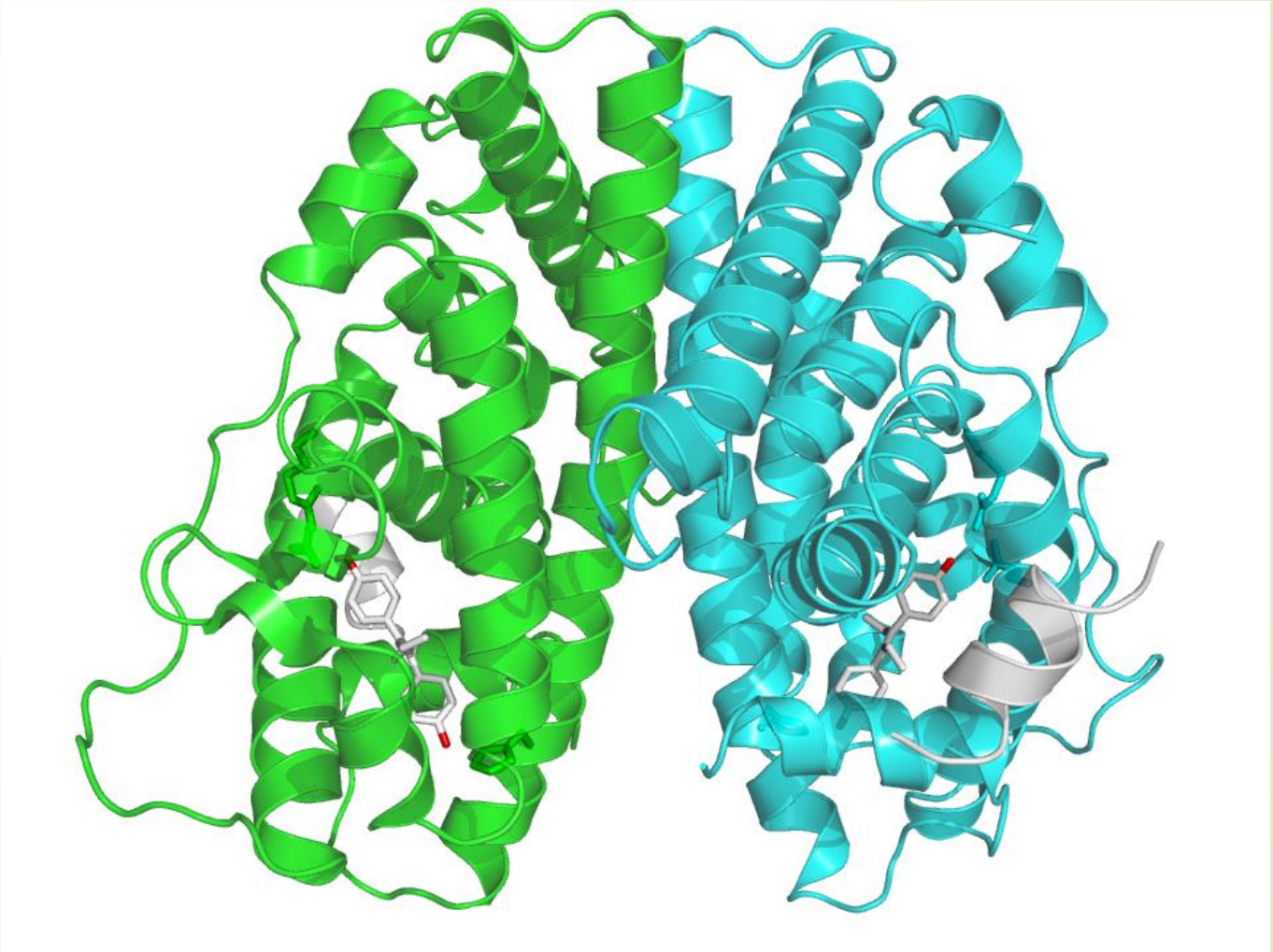
- Microarray gene expression profiling was used to identify genes normally silenced in ERα cells but derepressed upon exposure to the demethylating agent decitabine and resuppressed by gain of ERa activity/expression
- ERα dependent DNA methylation targets (n = 39) were enriched for ERa-binding sites, basal-up/luminal-down markers, cancer stem cell, epithelial–mesenchymal transition, and inflammatory and tumor suppressor genes
- Kaplan–Meier survival curve and Cox proportional hazards regression analyses indicated that these targets predicted poor distant metastasis–free survival(dmfs)
- The basal breast cancer subtype markers LCN2 and IFI27 showed the greatest inverse relationship with ERα expression/activity

implication

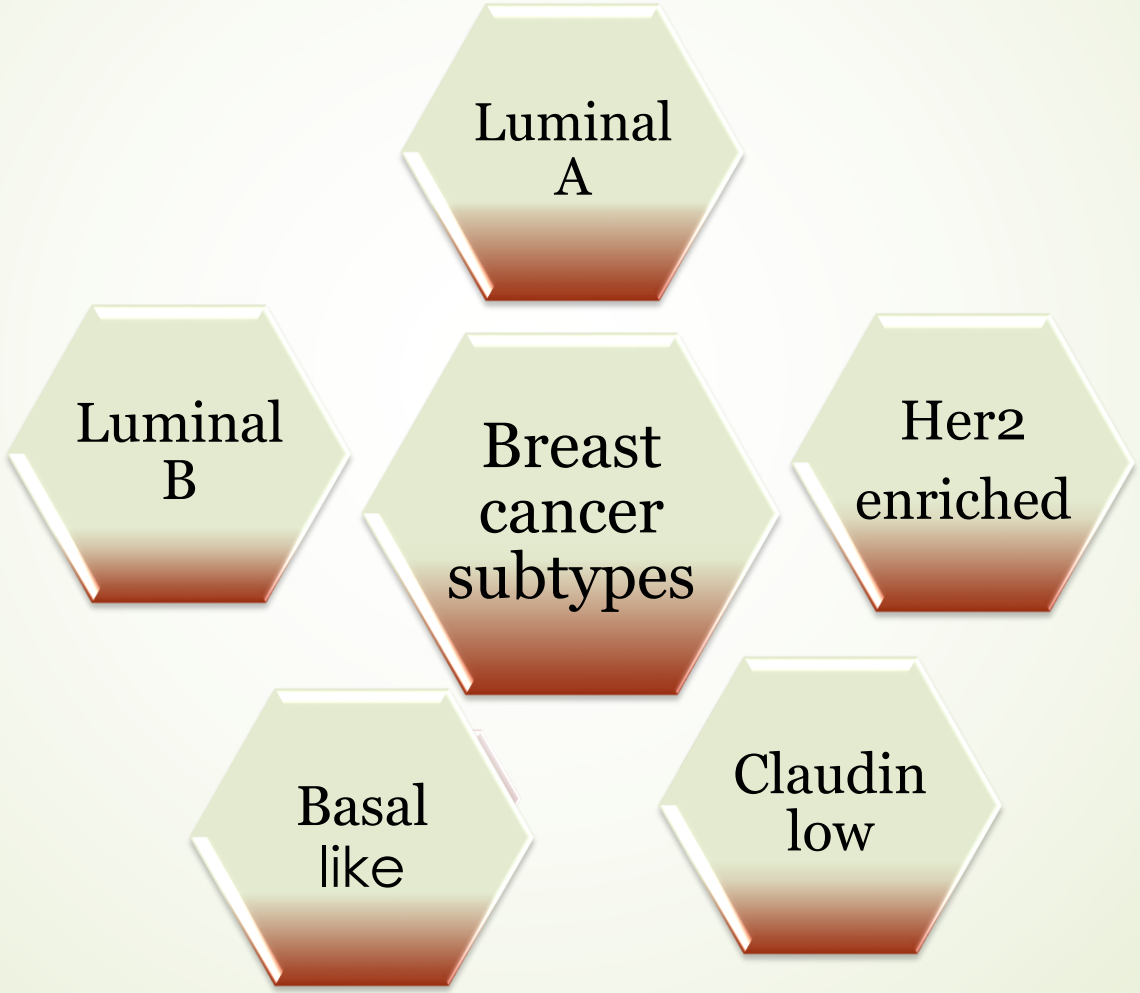
ERα directs DNA methylation–mediated silencing of specific genes that have biomarker potential in breast cancer subtypes

Introduction

ER(estrogen receptor Alpha)

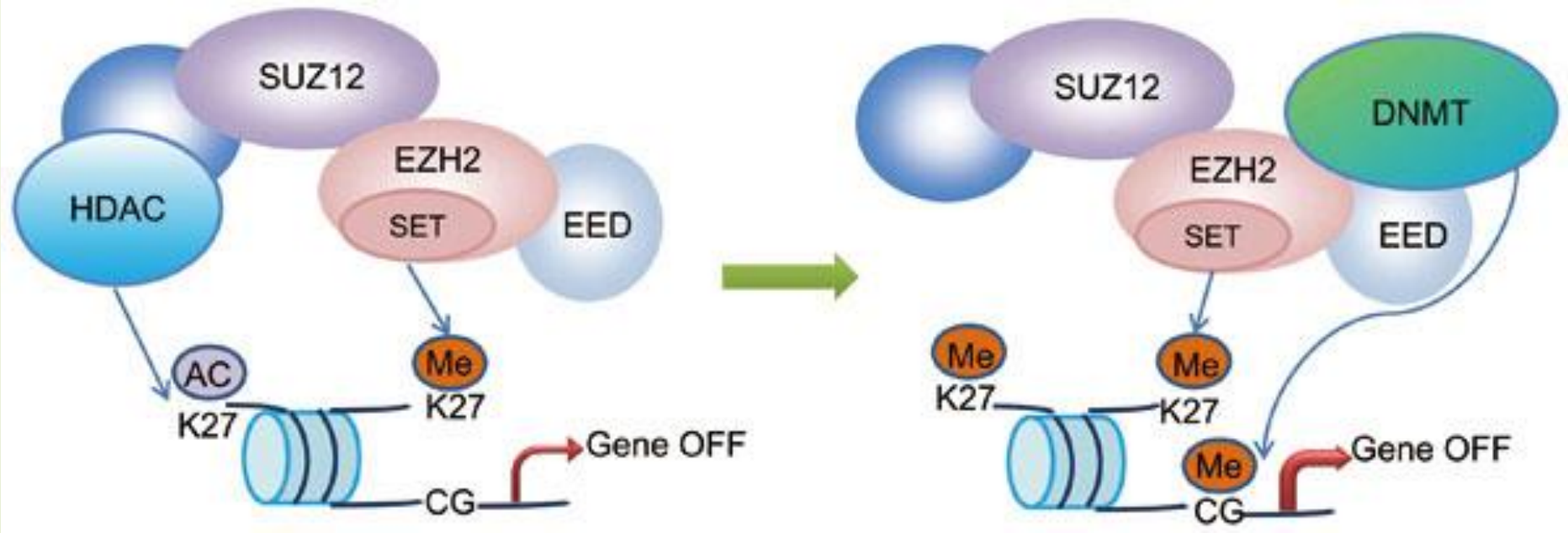


Presence of ERα lead to luminal classification



Introduction

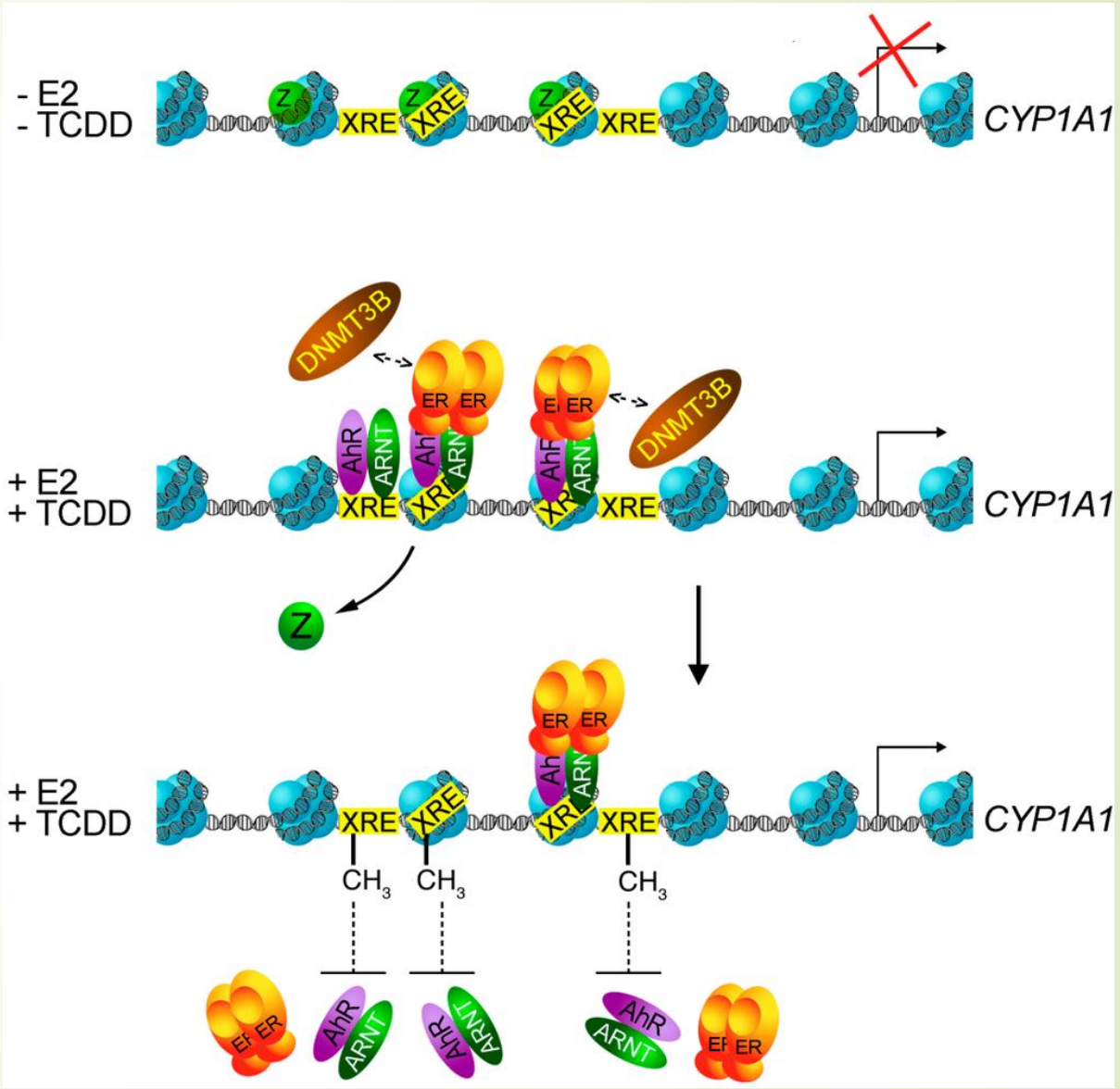
regulate gene expression



introduction

A functional link between ERα and DNA hypermethylation has been demonstrated at the CYP1A1 locus, whose gene product converts 17β-estradiol (E2) into a metabolite that inhibits proliferation; ERα silenced CYP1A1 by recruiting DNMT3B

The relationship between ERα and cyp1a1



To identify ERα targets CPG methylation mediated silencing by :

1. genes upregulated by the demethylating agent decitabine
2. genes upregulated by loss of ERα expression in a series of anti hormone -resistant T47D and MCF7 cell lines
3. Genes downregulated by E2 reexposure or increased ERα expression in anti hormone-resistant T47D and MCF7 cells
4. ER α for silencing and DNA methylation of the basal breast cancer subtype markers LCN2 and IFI27 in wild-type and anti hormone-resistant T47D based cell lines

Materials and methods

- The fulvestrant (FUL)–resistant cell lines (T47D/FUL, MCF7/FUL)(in estrogenized media (RPMI1640 plus 10% whole FBS) supplemented with 100 nmol/L fulvestrant)

- the estrogen deprivation (ED)–resistant cell lines (T47D/ED1, T47D/ED2) (in estrogen-free media (phenol red-free RPMI1640 plus 10% dextran-coated charcoal-stripped FBS)

were generated by continuous culture (8 weeks to >1 year) of wild-type T47D and MCF-7 cells

All authenticated by gene expression microarrays

Materials and methods

Cell lines

The lentiviral cell lines , T47D/ED1/VC, T47D/ED1/VC+E2, T47D/ED1/ER α, and T47D/ED1/ER α +E2 were generated by infecting ERα T47D/ED1 cells with an ER α -expressing lentivirus or an empty vector control (VC) lentivirus

Infected cells were maintained in estrogen-free or in 1 nmol/L E2–supplemented medium for 12 weeks again 4 weeks later, infected cells were sorted for the lentiviral ZsGreen fluorescent marker using a Becton Dickinson cell sorter.

To produce the lentiviral vectors, ER α 's coding region was excised from pHEGO using Eco RI and inserted into the Eco RI site of the lentiviral vector pLVX-EF1a-IRES-ZsGreen1

Materials and methods

- RNA isolation

RNA was purified using Qiagen ‘s RNeasy Plus Kits

- Real time pcr

using AMV First-Strand cDNA Kit,
TaqMan Universal PCR Master Mix,
and a 7900HT Fast Real-Time PCR system

Materials and methods

Microarray

Genome-wide RNA profiling was carried out by the Genomics Facility at Fox Chase Cancer Center (Philadelphia) using Agilent's Human Gene Expression

Immunoblot analysis

Using RIPA buffer and 40 mg protein per lane
Blots were visualized using the Odyssey Infrared Imaging System

DNA methylation analysis by pyrosequencing

Genomic DNA was isolated using the DNeasy Blood and Tissue Kit (Qiagen) and treated with bisulfite

assayed the ratios of methylated cytosines to thymines

Materials and methods

Human breast cancer cohorts

Breast cancer data from The Cancer Genome Atlas (TCGA)

project were downloaded via the International Cancer Genome

Consortium (ICGC) data portal

Methylation data were retrieved for 1,013 patients, 967 of whom also had ERa status available.

The gene expression microarray cohort comprising 2,116 breast cancers was previously constructed

Materials and methods

Metagenes

To analyze the composite expression level of gene sets in a tumor, gene sets were represented as metagene scores, or single number summary values were determined the expression breast cancer cohort.

These scores represent a expression values of each gene in the gene set in individual tumors.

Metagene scores were generated by "eigenvector" of each gene set in each tumor using singular value decomposition (SVD)
The eigenvector produced by SVD was rescaled to a

score between 0 and 1, with 0 relating to the lowest composite

expression value for a gene set, and 1 relating to the highest

RESULT

1. Identification of genes inversely correlated with ERα expression/activity
2. Expression analysis of the candidate ER α DNA methylation gene set in breast cancer
3. Inverse relationship between LCN2 and IFI27 expression and ER α
4. Direct relationship between methylation of LCN2 and IFI27 and ER α
5. LCN2 and IFI27 expression and CpG methylation in breast cancer cell lines

Identification of genes inversely correlated with ERa expression/activity

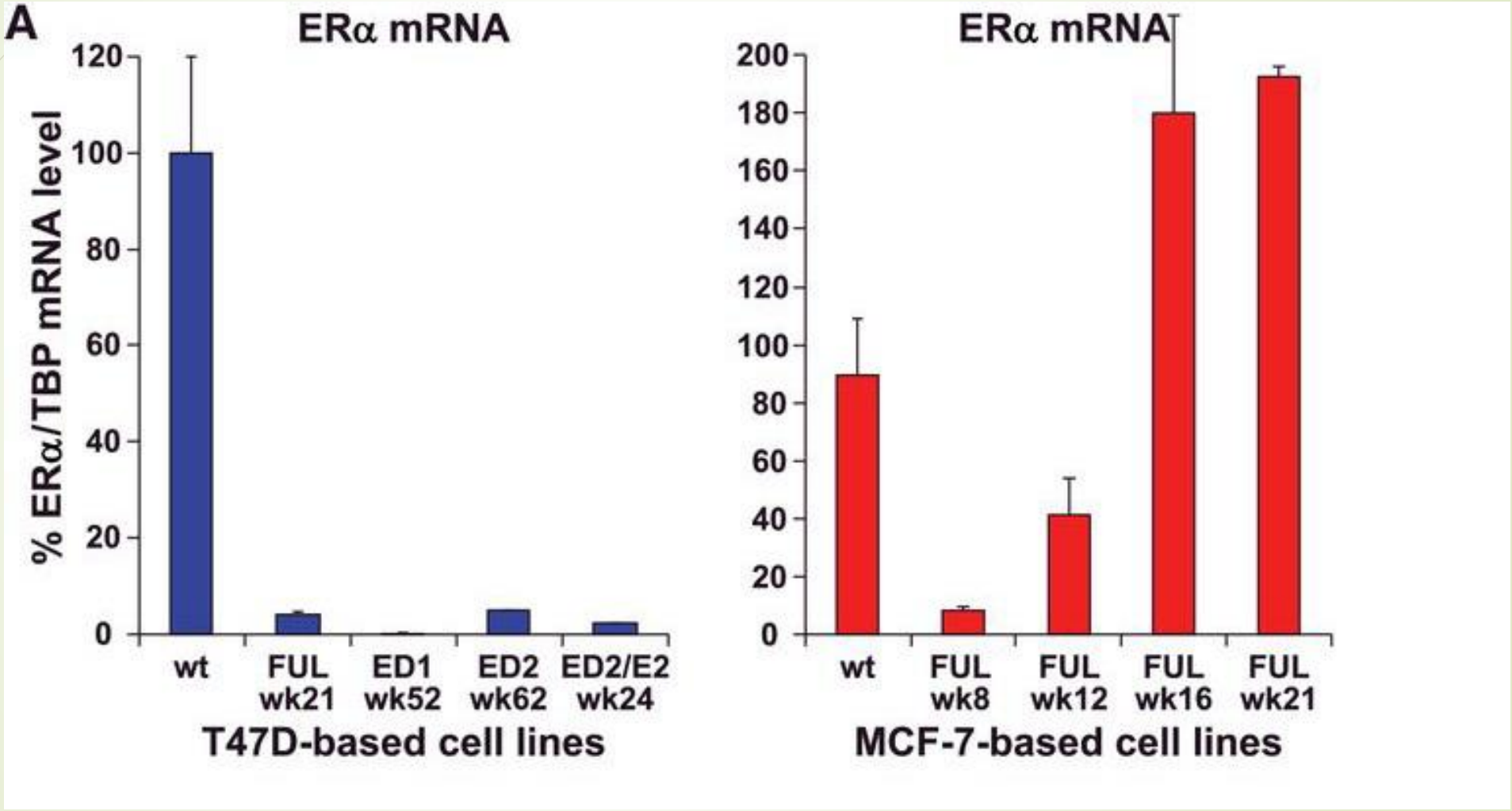
To identify ERα targets for DNA methylation–mediated silencing

1. those genes derepressed by loss of ER α expression
2. those genes resilenced by increased ERα activity or expression
3. those genes derepressed by loss of DNA methylation.

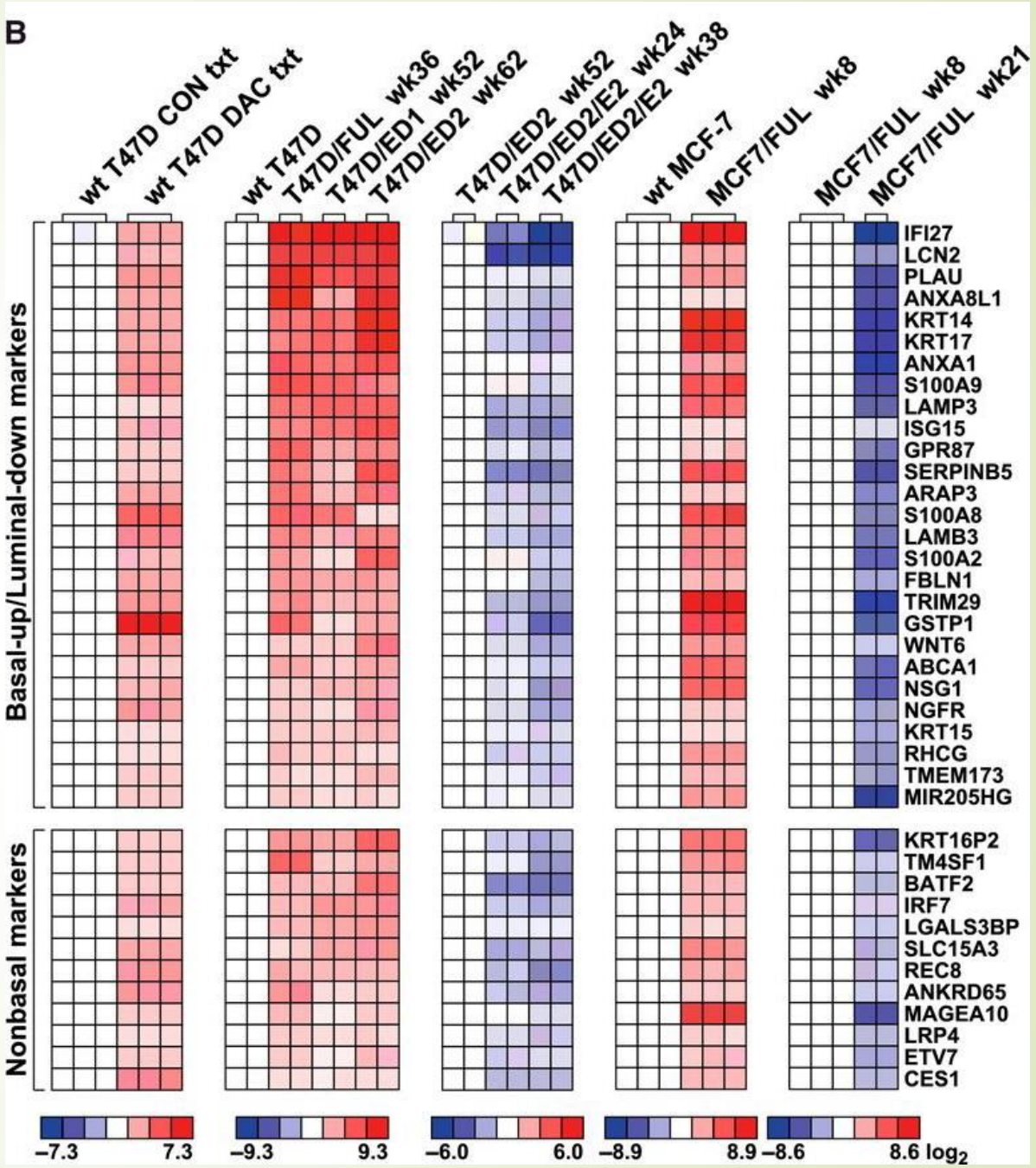
Identification of genes inversely correlated with ERα expression/activity

1. as acute depletion of ERα in estrogen-dependent cells leads to widespread cell death
2. microarrays
3. ERα mRNA levels were measured by qRT-PCR
4. Immunoblotting also demonstrated similar ERα protein losses
5. starting with wild-type ERα+ T47D and MCF-7 luminal breast cancer cells, a panel of ERα-low/negative T47D and MCF7 breast cancer cells was in 100 nmol/L fulvestrant or in estrogen-free media for 8 weeks to greater than 1 year
6. T47D/ED1 cells lost 99.9%, T47D/FUL and T47D/ED2 cells lost 95%, and MCF7/FUL cells (at week 8 of derivation) lost 90% of ERa mRNA compared with respective wild-type parental cells

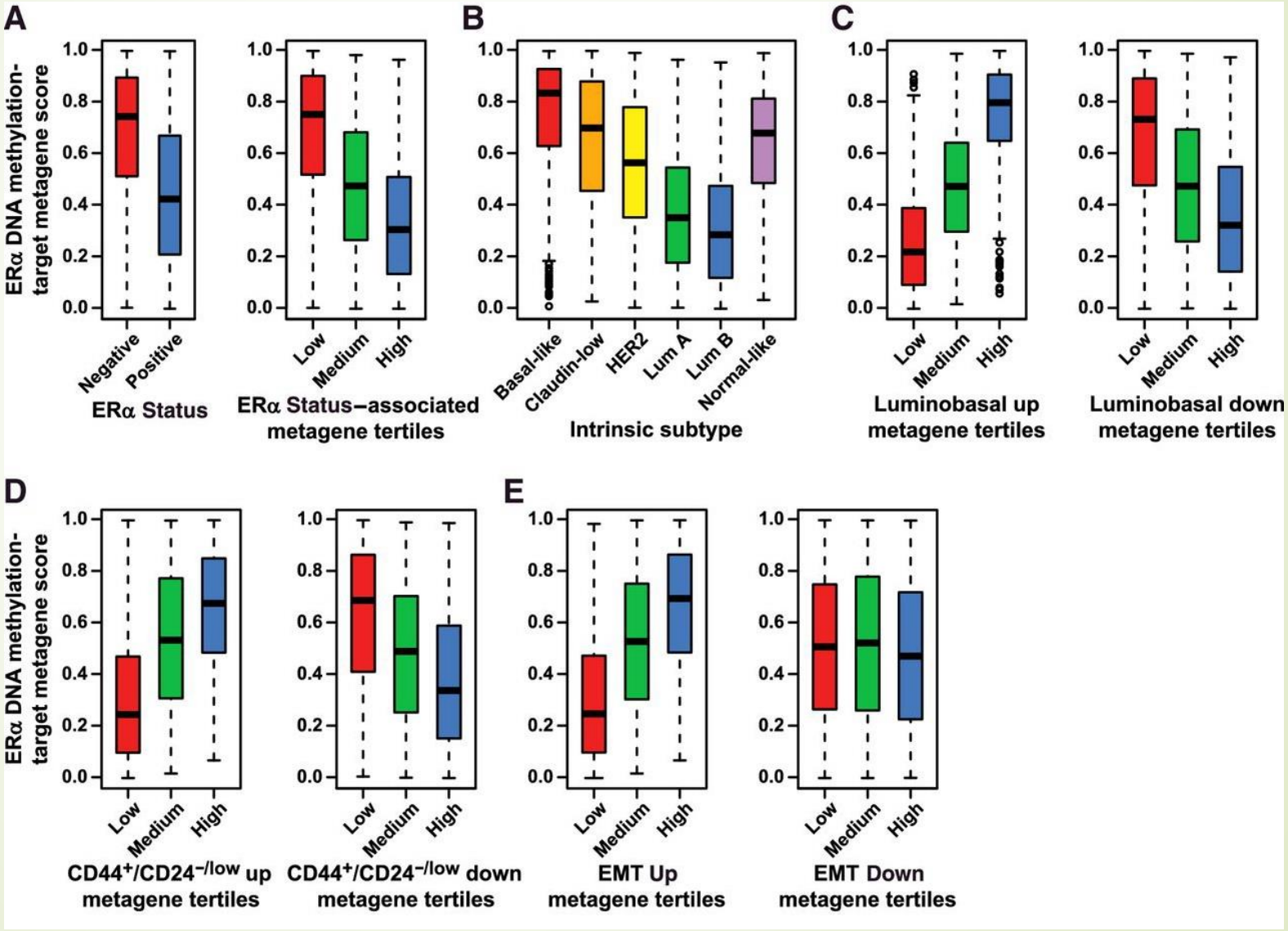
ERα mRNA level



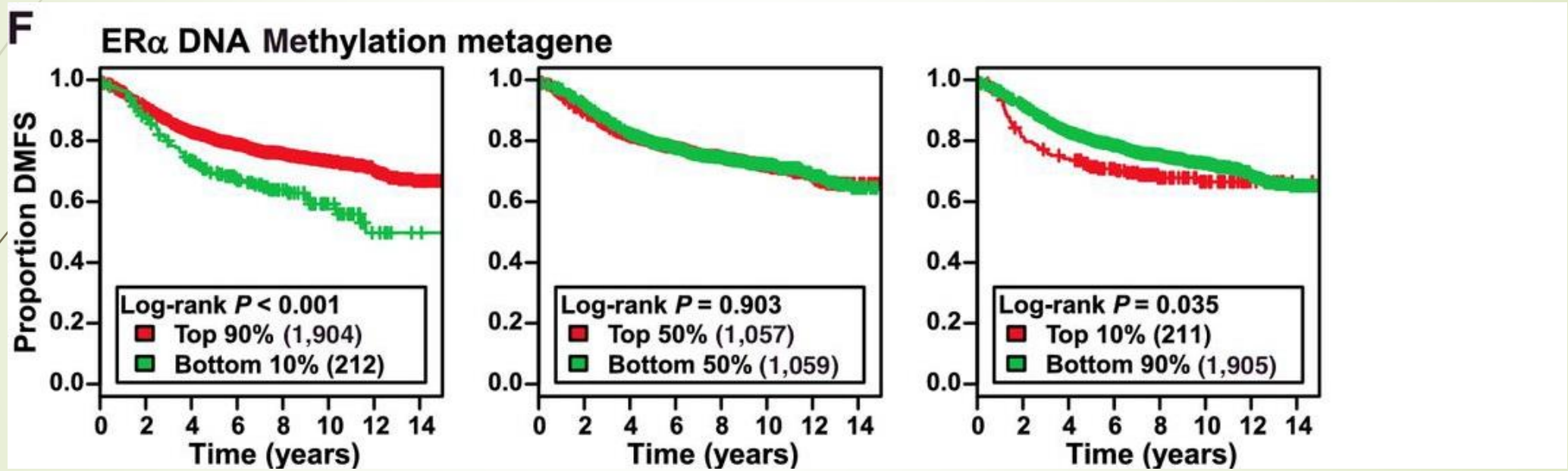
Microarray


B
wt T47D CON txt
wt T47D DAC txt
wt T47D
T47D/FUL wk36
T47D/ED1 wk52
T47D/ED2 wk62
T47D/ED2 wk52
T47D/ED2/E2 wk24
T47D/ED2/E2 wk38
wt MCF-7
MCF7/FUL wk8
MCF7/FUL wk8
MCF7/FUL wk21
Basal-up/Luminal-down markers
IFI27
LCN2
PLAU
ANXA8L1
KRT14
KRT17
ANXA1
S100A9
LAMP3
ISG15
GPR87
SERPINB5
ARAP3
S100A8
LAMB3
S100A2
FBLN1
TRIM29
GSTP1
WNT6
ABCA1
NSG1
NGFR
KRT15
RHCG
TMEM173
MIR205HG
Nonbasal markers
KRT16P2
TM4SF1
BATF2
IRF7
LGALS3BP
SLC15A3
REC8
ANKRD65
MAGEA10
LRP4
ETV7
CES1
−7.3
7.3
−9.3
9.3
−6.0
6.0
−8.9
8.9
−8.6
8.6 log2

ERα DNA Methylation



DNA methylation and DMFS



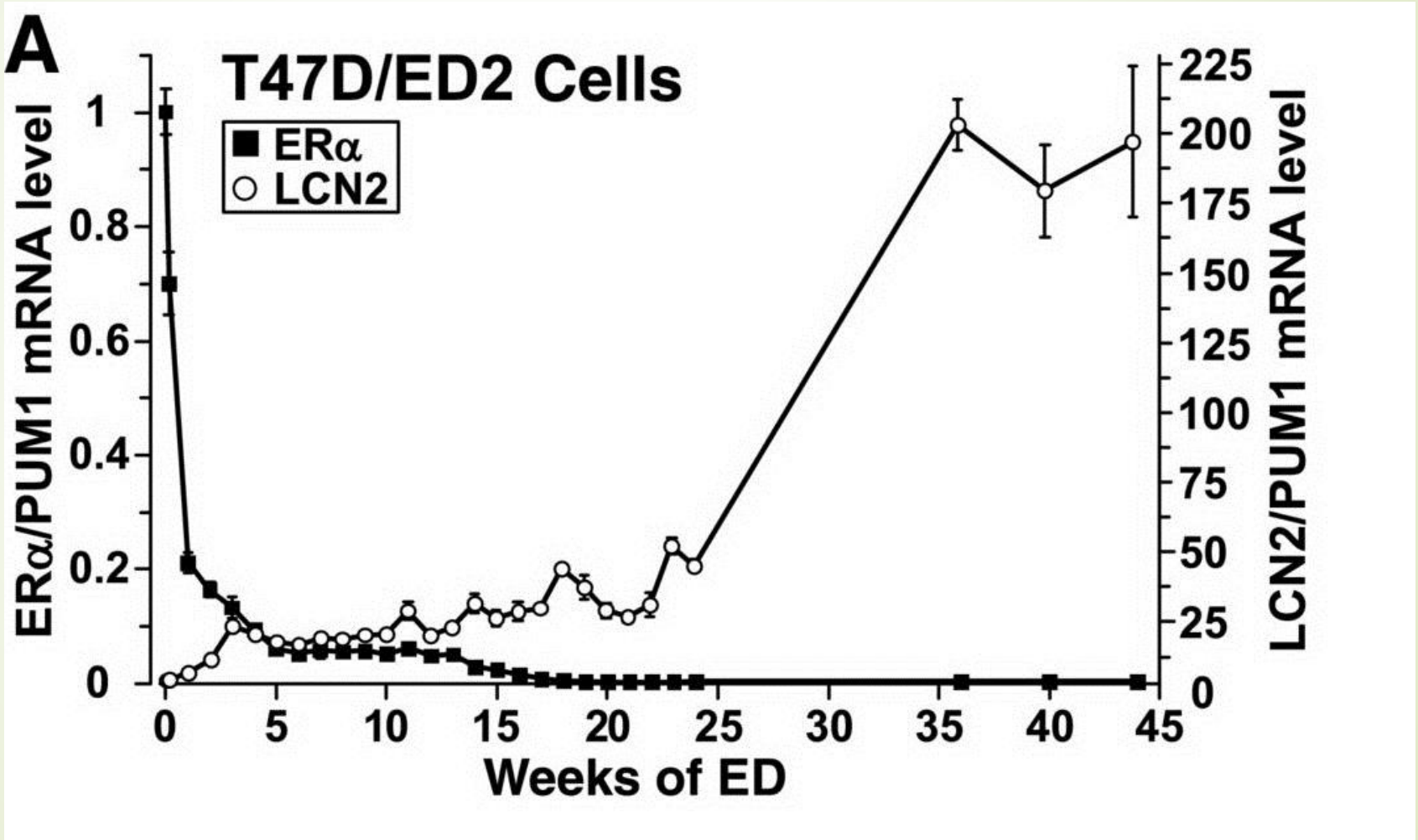
Inverse relationship between LCN2 and IFI27 expression and ER α

- LCN2 and IFI27 contain ERα-binding sites Also both are basal markers so LCN2 and IFI27 were selected for validation

of ERα-dependent changes in expression and 5mCpG levels.

- LCN2 mRNA and protein levels dramatically increased in a time-dependent manner after precipitous drops in ERα levels across all anti hormone-resistant models

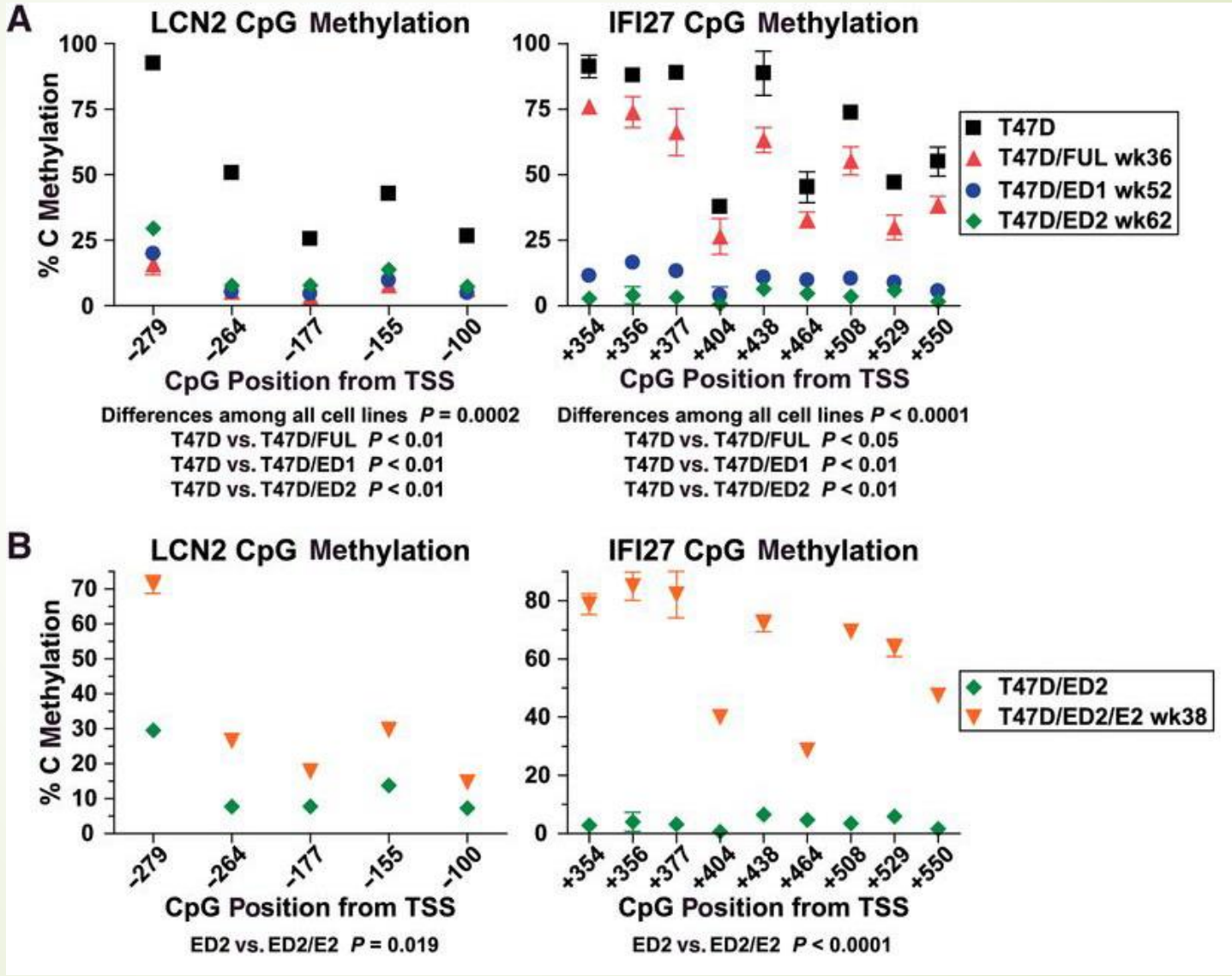
Inverse relationship between LCN2 and IFI27 expression and ER α



Direct relationship between methylation of LCN2 and IFI27 and ER α

- Levels of selected 5mCpG sites near the TSSs of LCN2 and IFI27 were quantitated across the T47D-based models by pyrosequencing
- This analysis found LCN2 and IFI27 CpG sites to be significantly hypo methylated in ERα negative T47D/FUL, T47D/ED1, and T47D/ED2 cells versus wild type ERα+ T47D cells and significantly hyper methylated upon E2 reexposure in T47D/ED2/E2 cells compared with parental T47D/ED2 cells
- Therefore, LCN2 and IFI27 CpG methylation levels directly associated with ERα expression activity.

Direct relationship between methylation of LCN2 and IFI27 and ER α



Direct relationship between methylation of LCN2 and IFI27 and ER α

- ERα-T47D/ED1 cells were infected with an ERα-expressing lentivirus or an empty VC lentivirus generating T47D/ED1/ERa and T47D/ED1/VC cells
- These infected cells were maintained with and without E2 for 12 weeks and subjected to two rounds of cell sorting for the lentiviral ZsGreen fluorescent marker.
- Characterization of these lentiviral cells lines demonstrated ERα mRNAs were expressed at high levels in ERa infected cells.

LCN2 and IFI27 expression and CpG methylation in breast cancer cell lines

D

LCN2: Correlation between CpG methylation and protein expression					
LCN2 CpG site	–279	–264	–177	–155	–100
Spearman ρ	–0.93	–0.91	–0.90	–0.92	–0.91
P value	<0.0001	0.0001	0.0002	<0.0001	0.0001

IFI27: Correlation between CpG methylation and RNA expression									
IFI27 CpG site	+354	+356	+377	+404	+438	+464	+508	+529	+550
Spearman ρ	0.07	–0.22	–0.53	–0.56	–0.68	–0.42	–0.74	–0.31	–0.67
P value	ns	ns	ns	0.038	0.013	ns	0.0064	ns	0.014

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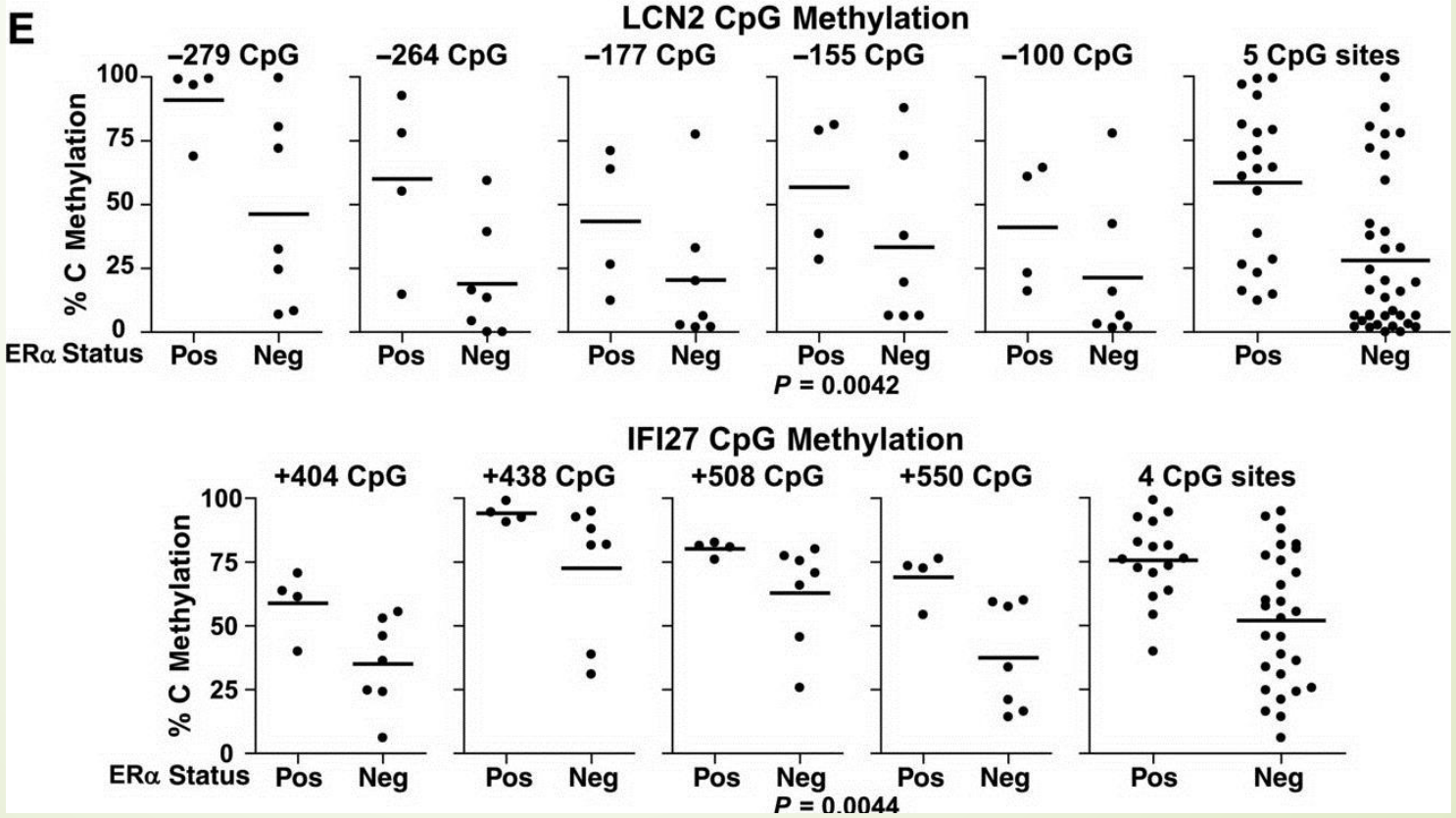

discussion

- As ERα plays such a pivotal role in a more favorable outcome in breast cancer, genes targeted by ERa for DNA methylation–mediated silencing likely play important roles in disease progression
- Taken together, our data indicate that ERα can silence genes via DNA methylation, such as LCN2 and IFI27. Moreover, ERα may direct DNA methylation–mediated silencing of a subpopulation of basal markers, CSC, and EMT genes that may potentially enforce luminal differentiation of breast cancer cells.

Thanks for your attention